A DISC ELECTROPHORETIC ANALYSIS OF THE NUCLEAR PROTEINS OF RAT LIVERS AFTER INJECTION OF THIOACETAMIDE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION AND REVIEW OF THE LITERATURE

Thioacetamide is a weak carcinogen that causes unusually dramatic changes in cells immediately after the beginning of treatment. In rats treated with thioacetamide, the parenchymal cells of the liver undergo rapid nuclear enlargement, develop cytoplasmic inclusions, and show hypertrophy of the nucleolus (Salomon et al., 1962).

Laird (1953) found that during the first four days of feeding thioacetamide the average cell dropped to two-thirds of its normal weight, and that there was a slight increase in cell number. Food consumption was low during this period. During the next few days the average cell weight increased rapidly (food consumption was improving) reaching and remaining at a normal level from the eleventh to the seventeenth day. Between the seventeenth and twenty-eighth days extensive cell proliferation resulted in a doubling in the number of cells in the average liver, and a reduction in the cell size until the cell weight was approximately one-half of the normal weight.

Volumetric studies by Kleinfeld and Koulish (1964), with regard to rat liver cells, indicated that the nucleus increased two-fold, the nucleolus increased fourteen-fold, and the cytoplasmic volume increased by only 20 per cent.

Upon attempting to ascertain whether changes in the nuclear volume of the rat cells were due to polyploidy or to

an increase of non-chromosomal components, Laird (1953) found that the amount of nuclear DNA remained constant upon treatment with thioacetamide whereas a definite change occurred in the amount of RNA and protein present. Although a good deal of research has been done on RNA variation in the nucleus, little has been done with nuclear protein. This thesis was designed to determine what, if any, changes occur in soluble liver cell proteins of rats exposed to thioacetamide.

During the first four days of Laird's experiment the cytoplasmic RNA decreased (excluding that in the supernatant) whereas nuclear RNA increased. Days four through seventeen showed an increase in cytoplasmic RNA (most notable in the supernatant fluid fraction) with the nuclear RNA reaching its maximal value on the eleventh day, at which time it was 3.5 times the corresponding control value. After twenty-eight days of treatment cytoplasmic RNA was at its lowest level, and although nuclear values were reduced below maximum quantities they remained distinctly higher than the control values.

Kleinfeld and Koulish (1964) found that under conditions of thioacetamide-stimulated RNA synthesis, the nuclear-nucleolar RNA turnover in the rat was, in part, independent of the cytoplasm. The thioacetamide-stimulated nucleus increased three-fold in RNA turnover (labeled with cytidine H^3), the nucleolus increased thirty-fold in RNA turnover, and the cytoplasm displayed a turnover equal to that in the control. The contrast between stimulated RNA turnover in the nucleus

and in the nucleolus compared to a normal turnover in the cytoplasm, suggests that the nuclear-nucleolar loss of label does not represent exclusive passage of formed nuclear RNA to the cytoplasm.

In 1965 Kleinfeld found that after incorporation of cytidine H^3 into the nucleolus of the rat liver the ratio of peak nucleolar radioactivity (at five hours) to the leveling off value (fifteen to eighteen hours) was about 3:1. Thus, not only are increased amounts of nucleolar RNA being synthesized but increased amounts are being lost from the nucleolus.

Busch and Adams (1965) discovered a marked change in nuclear i-RNA (RNA released into the aqueous phase by phenol in a fifteen minute incubation at 65°C) and residual RNA (RNA not released into the aqueous phase by phenol at 65°C) from rat liver cells treated with thioacetamide. In these RNA fractions there was a definite increase in the content of guanine and cytosine and a decrease in adenine and uracil. Since ribosomal RNA is rich in guanine and cytosine it would seem that nuclear increase reflects a decreased transport of the ribosomal RNA or an increased formation of it. Increased formation is indicated by an increase in total amount of this RNA and total uptake of isotope into nucleolar fractions. Evidence for increase in nucleolus activity comes from studies showing an increased RNA polymerase activity in nucleoli. Since increased biosynthesis of nucleolar RNA is correlated with decreased cytoplasmic ribosomes and the nucleolus has

been implicated as the ribosomal source, it seems there is an increased destruction of the RNA biosynthesized in the nucleolus as it proceeds through the nucleus to the cytoplasm.

Nucleolar formation of RNA and destruction of this RNA in the nucleus are fairly evident at this time. However very little is known about how thioacetamide affects the proteins of the nucleus. Laird (1953) found that by the fourth day of thioacetamide treatment cytoplasmic constituents were decreasing whereas nuclear proteins were increasing in quantity. Days four through seventeen showed protein increase in both the cytoplasm and nucleus. Nuclear proteins reached their maximum value on the eleventh day and were 2.5 times the control values. By the twenty-eighth day cytoplasmic proteins were lower than they had ever been and although nuclear proteins had dropped in quantity they were still higher than the controls.

Hafner (1967) investigated protein variations in thioacetamide-affected rat liver cells. Since nuclei which have been isolated in aqueous medium lose some loosely-bound water soluble proteins (Chaveau, Moule, and Rouiller, 1959) he chose to isolate the nuclei with a nonaqueous freeze-dry technique (Swanson et al., 1970). Hafner found distinct differences in the basic protein patterns and lesser variations in the acidic protein patterns of normal and neoplastic livers. In the basic protein patterns this consisted of an increase of two protein components (five basic proteins were in the controls

and seven were in the experimentals). Fifteen experimental and fifteen normal rats were used with five months of treatment. Time lapse in the experiment was to long for any analysis of progressive protein changes, and electrophoretic results on protein variation were to erratic for drawing any definite conclusion on specific linear changes.

In 1968 Meyer (personal communication) tried to alleviate the time lapse problem by sacrificing rats after 4, 14, 30, and 60 days. Although he used the same nucleus seperation technique Hafner had used, Meyer had difficulty getting pure samples of nuclei. By using whole cell proteins he found that acidic proteins were more heavily concentrated than basic proteins (visual observation). Also it was noted that basic proteins had increased in quantity during the first four days and then showed a quantity decrease at every following examination date. Acidic proteins were found to increase constantly in quantity until the sixtieth day at which time a normal amount of protein was recorded.

The method by which thioacetamide causes nuclear variation is unknown, but studies with thioacetamide-^{35}S have shown that instead of binding with proteins or alkylating nuclei acids, the compound is rapidly broken down since no increase in radioactivity is found in livers of rats fed thioacetamide ^{35}S (Nygaard, Eldjarn, and Nakken, 1954; Maloof and Soodak, 1961). Rees, Rowland and Varcoe (1966) studied the metabolism of thioacetamide with ^{3}H on the methyl group

and found that the carcinogen is metabolized in twenty-four hours through the liver and the kidneys. The liver was three times as active as the kidneys in converting the thioacetamide to acetamide. Rees et al., (1966) believed that this step was preliminary to the toxic and carcinogenic effects of thioacetamide in the liver.

This research project is designed to present an insight into what actually happened to the nuclear proteins of rat liver cells after they were treated with thioacetamide. The experiments were planned to deal with fewer proteins, a simpler and more dependable method for nuclear isolation, and a shorter time period, in order to avoid the problems encountered by previous workers.

METHODS AND MATERIALS

Thirty Sprague-Dawley rats, ranging in size from 200 to 550 grams, were obtained from the Iowa State University nutrition lab (Ames, Iowa). Five were males, twenty-five females. Throughout the experiment the rats were maintained on a diet of Purina lab chow, with an ample supply of water.

Daily injections of thioacetamide in isotonic Na Cl (6 mg/200 grams rat) were given subcutaneously to twenty-five rats. Five control rats, which had been randomly selected, received injections of the saline alone. Rats were killed between 8 AM and 9 AM on days 2, 4, 6, 8, and 10.

Rats were anaesthetized with medical ether and then decapitated. Upon removal all livers were weighed and liver volumes were determined. Nuclei were isolated using a slightly modified version of a technique developed by Bloebel and Potter (1966). Livers were placed in a cold Dounce homogenizer (Kontes Glass Company, Vineland, New Jersey) along with two volumes of .25 M sucrose in TKM (.05 M Tris HCl, .025 M KCl, .005 Mg Cl^2). Then the liver was homogenized with ten strokes of a plunger with a 156 micron clearance. This brei was thoroughly mixed with two volumes of 2.3 M sucrose in TKM.

Twenty ml of the final brei was placed in an ultracentrifuge tube and 2.3 M sucrose in TKM was layered under the brei with a long needled syringe. The tube was then placed in the head (F 50.1 fixed angle aluminum) of a Beckman L340 centrifuge and run at 123,000G for forty-five minutes. Following centrifugation the nuclei were concentrated at the bottom of the tube in a pellet; sucrose and debris were aspirated off. The inside of the tube was cleaned with tissue paper. Five ml of .01 M Tris HCl buffer solution (pH 7.5) was added to the nuclei. The sample was shaken once and refrigerated for six hours. At this time the sample was examined with a phase contrast microscope. Then the sample was recentrifuged at 3,000G and the protein sample (within the supernatant) was poured into a separate tube and refrigerated until use (two to twenty-four hours later). Ultraviolet absorbance of the protein sample at 260 nm and 280 nm

was determined with a Beckman DB spectrophotometer. By using the following formula protein concentrations for the samples could be determined:

Protein conc. (mg/ml)=F x 1/d x optical density at 280 mμ where d is the cuvette width (cm) and F is the ratio of the samples optical densities at 260 mμ and 280 mμ.

Protein samples were studied by disc gel electrophoresis. The procedure recommended by Tiselius, Stellan, and Jerstedt (1965) was used with a few modifications. Gels were prepared by mixing the listed solutions in the following proportions: Solution A - 1 part, Solution B - 2 parts, deionized water - 1 part, catalyst - 4 parts.

Chemical makeup of the solutions are as follows: Solution A - (1) 4.8 grams Tris/100 ml deionized water (2) .12 ml Tmed/100 ml deionized water (3) titrated with HCl to a pH of 7.5, Solution B - (1) 28 grams acrylamide/100 ml deionized water (2) .735 mg Bis (3) water to total 100 ml, catalyst - .14 gram ammonium persulfate/100 ml deionized water, Buffer - .1 M Tris at a pH of 7.5.

The electrophoretic apparatus used in the study was a Canalco Model 6 bath, with a Gelman power source. A standard electrophoretic run began with thirty minutes of "clearing current" at five milliamps/tube. Then the power source was turned off and .2cc of protein solution with .1 gram of sucrose (for increasing the density) was carefully layered on top of each gel tube (7 cm in length, .5 cm diameter - 6 cm

is filled with gel and 1 cm is left empty so the protein can settle in). Electrophoresis at 2.5 milliamps/tube was maintained for 45 minutes. At this time the voltage was increased to five milliamps/tube for five hours and fifteen minutes.

Upon completion of the run, gels were loosened from the gel tube by squirting cold buffer around them with a fine needled syringe, and they were removed. The gels were then dyed with Buffalo Black (.25 grams/100 ml of 7% trichloroacetic acid) for six hours. The same dyes were reused for all gels. After dying, the gels were destained with 7% acetic acid by allowing them to soak overnight in 200 ml of the solution.

Stained gels were then observed by two different methods visually and by one method mechanically. The first visual observation consisted of a complete count of the protein bands on each of the readable gels. Averages were determined for the number of protein bands in each sample of nuclei (three gels were made for each sample) and the averages were calculated for all the rats in one killing period. The second observation was made by measuring off a one inch block of gel, dividing this block into .25 inch sections, and counting the number of bands in each of the parts. In this manner slow and fast bands could be compared more readily.

Mechanical observations were made with a Gelscan Automatic Recording and Integrating Scanner (Gelman Instrument Co., Ann Arbor, Michigan) which was preset at the following readings; filter - red, slit width - 3 mm, slit length - .2mm.

Although it was impractical to try to determine the number of bands present from the scans (the bands were so close together at times that the scanner would miss one which could be visually observed), the proportional quantity of protein present could be determined. Graphical heights were determined at the starting point of the gels and at 3, 6, and 9 centimeters respectively. Proportions were then determined for the starting point (first peak) as compared to the other three distances. These proportional representations of protein were then averaged for each duration of treatment at each of the three distances. These averages were then graphed to show the quantitative changes in both the slow and fast proteins throughout the experimental period.

DATA AND DISCUSSION

The rats used in this experiment were observed daily. A general decrease in weight was found to occur following injections. The controls showed an average weight decrease of only 15 grams with no particular pattern being apparent in the decrease. The experimentals showed weight losses of 10, 19, 56, 68, and 121 grams for each of the four killing periods respectively. Some experimental rats lost hair around the injection area, and two experimental rats developed cases of diarrhea.

Although liver size remained fairly constant (average weight - 10.2 grams/rat) visual observation of nuclear pellet

size indicated a definite decrease in the second, third, fourth, and fifth killing periods. Nuclei and nucleoli were also observed with the phase contrast microscope, and it was noted that the typical thioacetamide poisoning symptoms (Rather, 1951; Kleinfeld, 1957) were present. The nuclei and nucleoli had increased in size and the nuclei contained some dark granules. All samples obtained seemed to be free of debris.

Spectrophotometer readings were made to determine the protein concentration of each sample obtained. These concentrations were found to average 3.4 mg/ml and had a range of 1.3 to 6.5 mg/ml. Generally there was little change in protein concentration throughout the experiment, and there were no patterns in the minor fluctuations which had occurred.

Two types of visual observations were made. First each gel was checked for total protein bands and the averages were calculated for each killing period (Table 1). Results from this seemed to indicate that very little change, if any, occurred in the number of proteins present in the treated nuclei. Second, visual observations were made to compare slow and fast bands readily (Table 2); there was little variation in the number of proteins in either group.

Dyed proteins showed readily in all but the last group of gels (fifth killing period). Due to the reusing of dye this final run produced gels which were poorly stained and consequently inadequate for analysis. Because of this, the

TABLE 1. Average number of protein bands per nuclear sample (one sample per rat) from rats treated with thioacetamide.

Days of Treatment	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Average
0	10	10.7	10.5	12		10.8
2	12	11	9.5	10	10	10.5
4	11	13	11.5	11.3	12	11.8
6	13	13	11.5	11	11.5	12
8	13	12	10.5	10	12	11.5

TABLE 2. Number of protein bands per section (four quarter inch sections comprising a one inch block) gel containing electrophoretic separations of nuclear protein from livers of rats treated with thioacetamide.

Days of Treatment	Section of Gel	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
0	first	6	5	5	*	*
	second	3	3	3		
	third	2	2	3		
	fourth	2	1	2		
2	first	6	6	*	7	*
	second	2	3		3	
	third	2	3		2	
	fourth	2	2		2	
4	first	5	*	8	5	5
	second	3		3	5	3
	third	3		1	1	1
	fourth	1		0	1	2
6	first	**	5	4	7	6
	second		4	3	2	3
	third		3	2	2	1
	fourth		2	2	1	0
8	first	6	7	4	4	5
	second	3	1	2	2	2
	third	1	3	2	2	1
	fourth	1	1	1	1	0

*Gels were broken. **Electrophoretic run was to long.

data covers only a nine-day period. Positively charged proteins are not included in the data since they usually moved as one diffuse band when separations were attempted. Occasionally bands of proteins seemed to be separated from this block. However, this was so erratic that valid conclusions could not be drawn.

As a final test for protein variation, all unbroken gels were mechanically scanned. Quantity of protein at any one point on the graph was evaluated and proportional averages for each killing period, at each of the three distances on the graph, were determined (Table 3). These averages were then graphed to show the quantitative changes in the fast, moderately fast, and moderately slow proteins (Figure 1).

The quantity of fast proteins (nine cm readings) increased after the first injection, were at the same level on the sixth day, and then decreased immensely by the eighth day. Moderately fast proteins (six cm readings) initially increased, dropped a slight amount on the fourth and sixth days, and dropped a great deal by the eighth day. The moderately slow proteins increased at first, increased slightly more on the fourth day, decreased a little on the sixth day, and drastically dropped on the final day. The quantity of very slow proteins increased constantly.

Although definite changes in the number of protein bands present were hard to ascertain due to the closeness of the bands and the quantity of bands present, evidence of

TABLE 3. Proportional comparison of protein concentrations (nuclear liver proteins isolated from rats treated with thioacetamide) at four distances of electrophoretic migration. Proportions determined by measuring graphical heights at the starting point and at three chosen distances and then comparing the starting point to the other three heights.

Graphical distance from Origin (cm)	Proportions for Experimental Rats and Controls Days of Treatment				
	0	2	4	6	8
3	2.2	1.9	1.6	2.1	4.7
6	2.4	2.1	2.2	2.1	4.3
9	5.4	5.4	3.3	3.3	7.7

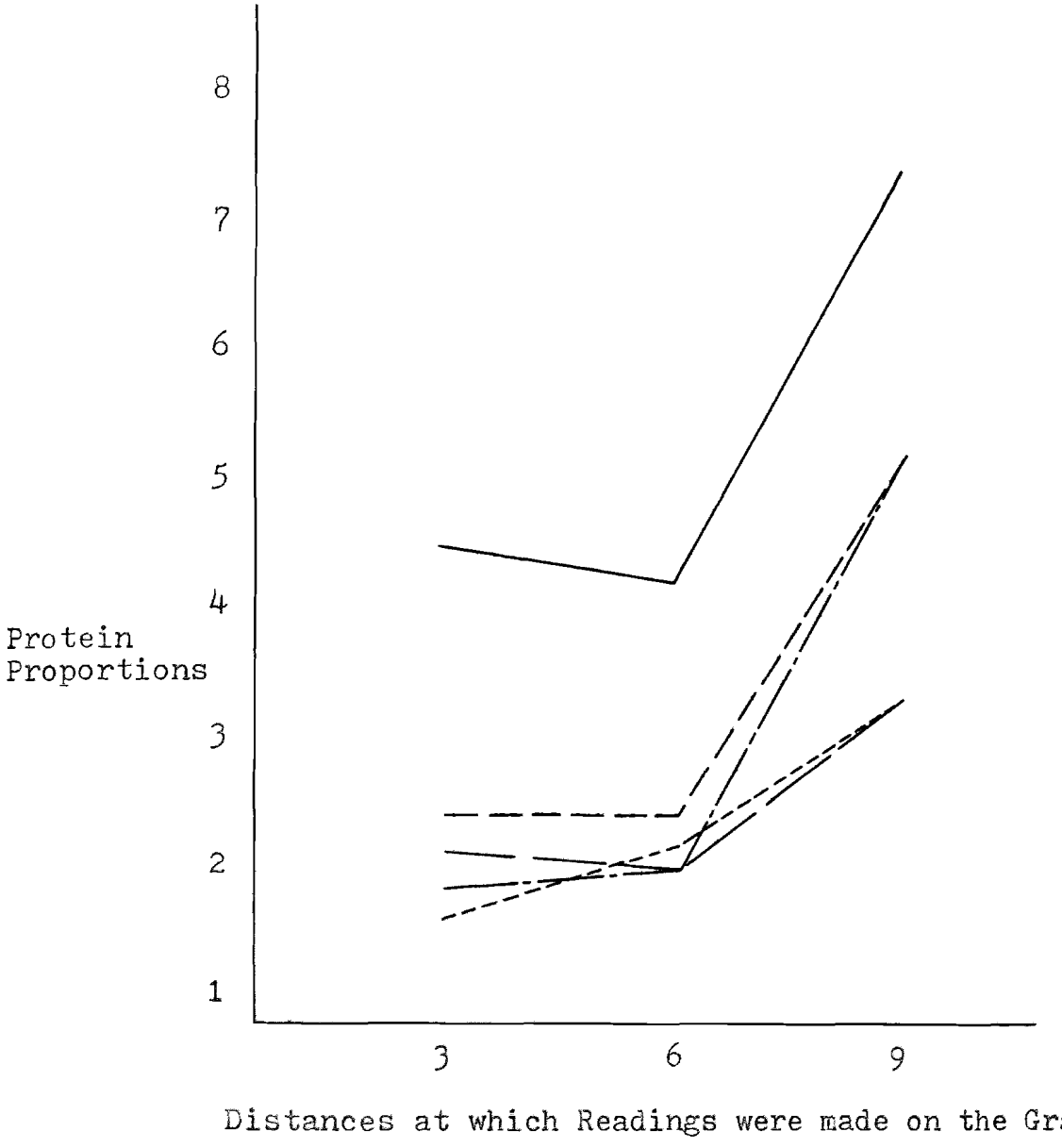

FIGURE 1. Proportional comparison of protein concentrations (nuclear liver proteins isolated from rats treated with thioacetamide) at four distances of electrophoretic migration. Key indicates number of days of thioacetamide treatment per group of rats. (1)— — — — — zero days (2)——·——·——two days (3)----------four days (4)—— ——·——six days (5)——————eight days

proportional quantitative changes was definite. Fast proteins seemed to increase initially a great deal and then drop off rapidly after six or seven injections. Moderately fast proteins were fairly high in quantity to start with, increased slightly, and then dropped off after six or seven injections. Moderately slow proteins were very high to begin with, increased even more, and then rapidly declined around the sixth or seventh day of injection. These proportional changes coupled with the knowledge that the protein concentration remains constant, show that the very slow (or non-moving) proteins seem to be increasing in quantity, while the fast, moderately fast, and moderately slow proteins decrease in quantity.

Although general microscopic observation agree with results reported in previous literature (Rather, 1951) this experiment showed definite variation from Laird's data (1953) on the total weight pattern of rats treated with thioacetamide. Laird noted that after the fourth day of injection (with amounts of thioacetamide equal to those used in this experiment) the rats started to approach a normal weight level. Meyer (personal communication) also found a constant weight gain. This research has shown the weight loss to increase consistently through the tenth day of treatment. This is perhaps due to differences in rat ages. All of Laird's and Meyer's rats weighed about 200 grams, were probably younger than those used in this experiment, and were in a period of growth rather than of stability or loss.

Protein concentrations were also found to differ from those recorded by Laird. Spectrophotometer readings showed relatively consistent concentrations of protein in this experiment. Laird (1953) had observed an early increase in protein quantity and then a slow decrease in amount until the twenty-eighth day when it was normal again. One possible reason for this might be that, since the data collection started on the second day of this project, the initial increase in protein concentration was not observed. The consistency in protein level for the remaining period of time is very possibly due to the rapid and consistent decrease in the weight of the experimental rats. This might be expected since starved animals fed on a low protein diet often show an increase in nucleolar size which has been shown to be caused by an increase in RNA and protein synthesis (Stenram, 1958; Kleinfeld, 1966). Another reason for the variation in data might be that the proteins were analyzed differently in the two experiments. Whereas Laird (1953) analyzed for protein by a nesslerization method which involved using whole cell nuclei this experiment used a Tris buffer solution to draw proteins from the nuclei and then the sample was analyzed with electrophoresis. Thus the quantity of easily extractable protein remained constant. Total nuclear protein probably declined along with the nuclear pellet size. This would indicate that a greater proportion of nuclear protein can be easily extracted from the nuclei of treated rats.

In analyzing the number of different proteins present in the normal and experimental rats, it was found that a larger variety of proteins was present than had been expected. Hafner (1967) had noted five separate proteins in the controls and seven in the experimentals. Due to the fact that he was observing the total number of proteins in nuclei, isolated by a non-aqueous method that would not allow loss of proteins, it was anticipated that fewer protein types would be obtained with the particular aqueous nuclear isolation technique used here. In this case, however, an average of 11 proteins were found in each sample of rat nuclei. The minimal number of bands counted was nine and the maximum was fourteen (counts being made by visually observing each gel). Hafner's isolation procedures produced much smaller samples of cell nuclei which might account for the difference, since components in low concentration might not have been detected.

Upon studying Figure 1, one can see that significant changes in protein pattern occurred between days zero and two and between days six and eight. Days zero through two showed an initial increase in the proportions of fast, moderately fast, and moderately slow proteins, whereas days six through eight showed a rapid decrease in all of these proportions and an increase in the amount of slow proteins present. This indicates that more study of protein change should be centered around these time periods. Perhaps shorter time periods would illustrate more clearly any changes in protein concentration.

Also better separation of proteins during electrophoresis would be helpful. Better separation could be obtained by allowing the electrophoretic runs to last longer or by varying the ingredients in the gel so larger proteins can pass through it more readily.

It is apparent from these variations in protein patterns and by the fact that protein concentration remains constant while the size of the nuclear pellet seems to diminish, that thioacetamide must be affecting the rate of increase in nuclear proteins (by synthesis or by uptake of cytoplasmic proteins) or the forces which hold the proteins in the nucleus. It is possible that proteins are in the nucleus because of salts binding to chromosomes. If this is so, then a change in the ionic character of the binding sites would lead to a change in the average charge of moderately extractable proteins, and a greater extractability.

Determination of protein concentration by Laird indicated that total protein from aqueously isolated nuclei increase in quantity. This increase seems to be due to protein synthesis, since both nuclear and cytoplasmic proteins increase in quantity after the fourth day. The initial decrease in cytoplasmic constituents and increase in nuclear proteins indicates that the synthesis is occurring in the nucleus or nucleolus.

Although no definite explanation can be suggested as to how thioacetamide caused this synthesis to occur, Laird (1953) has definitely shown that there is no increase in total amount

of DNA after treatment. The results of this study showed a decreased amount of the total fast, moderately fast, and moderately slow moving proteins. This suggests that thioacetamide might be responsible for the production of charged particles which bind to the proteins and consequently slow their electrophoretic migration rate. If this is the case, then the greatest amount of binding occurs between the sixth and eighth days of treatment. This would account for the high amount of "slow proteins" which were present by the eighth day of the experiment. It is speculated that this type of ionic attachment also could have significant affect on the nuclear DNA. A possible method for varifying this occurrence could be by separating the same proteins according to their isoelectric points. This can be done readily with isoelectric focusing and would clearly show the attachment of particles to the proteins. When particles would attach, new isoelectric points would form, with the proteins migrating to new destinations.

CONCLUSIONS

This investigation was designdd for studying the aqueously isolated proteins of normal and thioacetamide treated nuclei of rat livers.

Upon injection of thioacetamide, protein concentrations were found to remain relatively constant although visual observation indicated a definite decrease in nuclear pellet

size. Apparently a greater proportion of nuclear protein is extracted from nuclei treated with thioacetamide.

Electrophoresis showed an initial increase (days zero through two) in the quantity of fast, moderately fast, and moderately slow proteins and then a rapid decrease of the same groups between days six through eight. A possible explanation might be that thioacetamide assisted in the production of charged particles which bound to the proteins and consequently slowed their migration rates. It is apparent that further research should be centered around the time periods between days zero and two and days six through eight.

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